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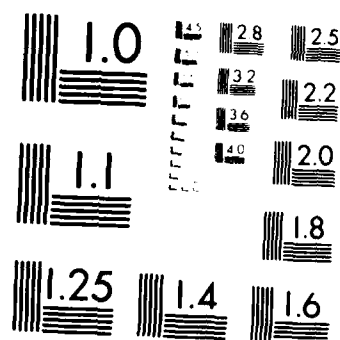
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NEUROPEPTIDES IN EXPERIMENTAL HEAD INJURY

ANNUAL REPORT

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Much of the damage resulting from ischemic or traumatic insults to the central nervous system appears to result from secondary injury mechanisms relating to the release of endogenous factors. Endogenous opioids may represent one such class of pathophysiological factors, and have been implicated in traumatic spinal cord injury, ischemic spinal cord injury and ischemic brain injury. The studies covered under the present contract examine the potential pathophysiological role of endogenous opioids and their receptor-mediated changes following traumaitc brain injury in both cats and rats. Utilizing a

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fluid percussion device manufactured for us by Medical College of Virginia, we have evaluated the effects of graded levels of injury on outcome measures, including mean arterial pressure, intracranial pressure, electroencephalographic (EEG) activity, and cerebral blood flow in the cat. In a continue of studies begun during the first year of the present contract, we have compared the effectiveness of the opiate antagonist WIN44,441-3, which has enhanced activity at the κ-opiate receptor, with its inactive stereoisomer WIN44,441-2, saline, and dopamine hydrochloride.

A moderate level of injury caused a brief period of hypertension (1 - 3 minutes), followed by a significant decrease in mean arterial pressure and whole brain blood flow (measured using the radiolabelled microsphere technique). Fluid percussion injury also caused a decline in EEG amplitude. Administration of the κ-opiate receptor antagonist WIN44,441-3, at 15 minutes following head injury caused a rapid and significant increase in mean arterial pressure and EEG amplitude that was sustained for up to two hours, with an associated increase in whole brain blood flow. Blood flow was also significantly increased in the midbrain, brainstem, cerebellum and basal ganglia where injury, evidenced by histological changes, was most severe. Dopamine administration improved post-traumatic MAP without affecting EEG or regional CBF, indicating that the efficacy of WIN44,441-3 was not due solely to its pressor effects. Administration of the inactive stereoisomer WIN44,441-2 or saline had no effect on MAP, EEG, or regional CBF following traumatic brain injury.

In addition, during the current year, we have utilized both a low and high-level model of head injury to examine the injury dependent alterations in regional endogenous opioid concentrations in the cat. Animals were injured at either low- or high-level of injury and brains were removed, sectioned and frozen for radioimmunoassay at two-hours post-injury (the time point where maximal changes in regional CBF occur). Changes in regional concentrations of endogenous opioids were compared and related to histopathological/blood flow changes. Our results demonstrate that dynorphin-, but not leucine-enkephalin-like immunoreactivity or β-endorphin-like immunoreactivity accumulate at injury sites following traumatic brain injury, suggesting a potential role for dynorphin in the injury process.

We have completed a thorough evaluation of novel parasaggital model of brain injury in the rat and have compared the physiological and cardiovascular response to this lateral injury model with a midline (vertex) injury model. Using the lateral injury model, we have also evaluated the therapeutic efficacy of nalmefene (a newly synthesized opiate antagonist with increased affinity at κ-receptors) and CG-3703 (a long-acting centrally active TRH analog). Both compounds were found to improve blood pressure, neurological outcome and (for CG-3703) survival following brain injury. Together, these results suggest that endogenous opioids contribute to the pathophysiology of head injury and indicate that opiate antagonists with increased activity at κ-sites or TRH analogs may be effective in the treatment of acute head injury.

Finally, we have initiated pilot studies utilizing nuclear magnetic resonance spectroscopy (MRS) to evaluate the dynamic in vivo metabolic response to traumatic brain injury in the rat. We utilized phosphorous (^{31}P) MRS to determine whether alterations in intracellular high-energy phosphates (ATP, phosphocreatine (PCr), inorganic phosphates (Pi)) and pH occur in response to fluid percussion injury. We observed that the PCr/Pi ratio decreased following moderate ($x = 2.0$ atm) injury with a simultaneous fall in intracellular pH. Although both PCr/Pi recovered to normal values by 100 min post-injury, PCr/Pi subsequently decreased again at 2 h post-injury without recovery over the succeeding 6 h. No changes in ATP were observed at any time. These studies have shown that transient changes in high energy phosphates and intracellular pH occur in response to traumatic brain injury. This decreased PCr/Pi ratio in the absence of hypoxia or tissue acidosis may be a reflection of mitochondrial dysfunction and may thus be a marker of irreversible tissue injury.

FOREWORD

In conducting the research described in this report, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 86-23, Revised 1985).

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SUMMARY

The studies covered under the second year of the present contract examine the potential pathophysiological role of endogenous opioids and their receptor-mediated changes following traumatic brain injury in both cats and rats. Utilizing a fluid percussion brain injury device manufactured for us by Medical College of Virginia, we have evaluated the physiological/cardiovascular response to brain injury, including mean arterial pressure, intracranial pressure, electroencephalographic (EEG) activity (Fast-Fourier transformed EEG), and regional cerebral blood flow (CBF) (as measured by radiolabelled microspheres) in the cat. We have completed studies, begun in the first year of the Army contract, which compare the effectiveness of the opiate antagonist WIN44,441-3 (WIN(-)), which has enhanced activity at the κ-opiate receptor, with its inactive stereoisomer WIN44, 441-2 and saline. Additionally, to evaluate whether the efficacy of WIN(-) were dependent solely upon its salubrious effect on blood pressure, a fourth group of animals have been treated with constant infusion of dopamine. Constant infusion of dopamine caused a significant and prolonged elevation in MAP which paralleled that of WIN(-). However, no improvement in EEG or regional CBF was noted following dopamine treatment. These results suggest that the efficacy of WIN(-) in enhancing post-traumatic regional CBF appears to be unrelated to any increases observed in MAP or ICP.

In addition, during the current year, we have utilized both a low and high-level model of head injury to examine the injury dependent alterations in regional endogenous opioid concentrations in the cat. Animals were injured at either low- or high-level of injury and brains were removed, sectioned and frozen for radioimmunoassay at two-hours post-injury (the time point where maximal changes in regional CBF occur). Changes in regional concentrations of endogenous opioids were compared and related to histopathological/blood flow changes. Our results demonstrate that dynorphin-, but not

leucine-enkephalin-like immunoreactivity or β-endorphin-like immunoreactivity accumulate at injury sites following traumatic brain injury, suggesting a potential role for dynorphin in the injury process.

We have completed a thorough evaluation of novel parasaggital model of brain injury in the rat and have compared the physiological and cardiovascular response to this lateral injury model with a midline (vertex) injury model. Using the lateral injury model, we have also evaluated the therapeutic efficacy of nalmefene (a newly synthesized opiate antagonist with increased affinity at κ-receptors) and CG-3703 (a long-acting centrally active TRH analog). Both compounds were found to improve blood pressure, neurological outcome and (for CG-3703) survival following brain injury. Together, these results suggest that endogenous opioids contribute to the pathophysiology of head injury and indicate that opiate antagonists with increased activity at κ-sites or TRH analogs may be effective in the treatment of acute head injury.

Finally, we have initiated pilot studies utilizing nuclear magnetic resonance spectroscopy (MRS) to evaluate the dynamic in vivo metabolic response to traumatic brain injury in the rat. We utilized phosphorous (^{31}P) MRS to determine whether alterations in intracellular high-energy phosphates (ATP, phosphocreatine (PCr), inorganic phosphates (Pi)) and pH occur in response to fluid percussion injury. We observed that the PCr/Pi ratio decreased following moderate ($\bar{x}$ = 2.0 atm) injury with a simultaneous fall in intracellular pH. Although both PCr/Pi recovered to normal values by 100 min post-injury, PCr/Pi subsequently decreased again at 2 h post-injury without recovery over the succeeding 6 h. No changes in ATP were observed at any time. These studies have shown that transient changes in high energy phosphates and intracellular pH occur in response to traumatic brain injury. This decreased PCr/Pi ratio in the absence of hypoxia or tissue acidosis may be a reflection of mitochondrial dysfunction and may thus be a marker of irreversible tissue injury.

TABLE OF CONTENTS

STATEMENT OF PROBLEM

It is clear that much of the neurological deficit which follows traumatic injury to the central nervous system (CNS) results not only from the immediate and direct effects of the trauma in severing neuronal connections, but from reductions of blood flow due to release of endogenous factors. It has preciously been established that endogenous opioid systems contribute to the secondary pathophysiological events in spinal cord injury by contributing to the reduction in spinal cord blood flow. Although work in this laboratory has suggested that endogenous opioids, acting at κ-receptors in the spinal cord, may act as pathophysiological factors in traumatic spinal cord injury, the response of physiologically active endogenous opiate systems to traumatic brain injury is unknown, and the possible role of endogenous opioid systems in the pathophysiology of traumatic brain injury is essentially unexplored. Such studies may lead to novel pharmacological therapies such as selective opiate-receptor antagonists (e.g. κ-receptor antagonists) or physiological opiate antagonists (e.g. TRH, TRH analogs).

BACKGROUND

It has been suggested that the endogenous opioids may contribute to central nervous system injury, in part by reducing blood flow to the CNS or by altering brain metabolic activity (1). Based on our work in experimental shock, we have previously demonstrated that traumatic cervical spinal cord injury in the cat caused an elevation of plasma β-endorphin-like immunoreactivity associated with reduction in spinal cord blood flow (SCBF) (2,3). Treatment with high doses of naloxone (2 mg/kg) in these studies significantly improved both SCBF and neurological recovery, This beneficial effect of high dose naloxone in spinal cord injury has been confirmed by other researchers (4-6). Since it is known that delta- and κ-receptors are less naloxone sensitive (7), the high

pharmacological doses of naloxone required in the spinal injury studies suggest that the naloxone effects may have been due to actions at such non-mu opiate receptors. To this end, we have demonstrated that the dynorphin family of opioids in unique in producing dose-related hindlimb paralysis following intrathecal administration in rats (8). Since dynorphin is proposed as the endogenous ligand for the κ-receptor, this observation suggested that the dynorphin/κ-receptor system may be involved in the pathophysiology of spinal cord injury. Dynorphin A-immunoreactive materials (Dyn A-ir) (1-17), but not leu-enkephalin, met-enkephalin or Dyn A (1-8), was found to be increased at the injury site following experimental traumatic spinal cord injury in the rat(9). It has also been observed that the opiate antagonist WIN44 441-3, which has increased activity at κ-sites, stereospecifically improves neurological recovery after traumatic spinal injury in the cat and ischemic spinal injury in the rabbit (10). Since WIN44,441-3 was approximately 50 times more potent than naloxone in its therapeutic effects in the rabbit, the relative dose-response effects are consistent with a κ-receptor mechanism of action (11).

Although the physiological changes following head injury that have been related to clinical outcome include changes in blood pressure, hypoxia and ischemia (12-14), little is known concerning the role of endogenous opioids in mediating the pathophysiological sequelae of head trauma. Naloxone treatment, at high doses (2 mg/kg), has been found to significantly improve the cortical somatosensory-evoked response and prevented the areas of multifocal low blood flow and infarction following ischemic brain injury produced by air embolism in the dog (15). Hayed at al. recently demonstrated that high doses of naloxone treatment improved blood pressure, brain perfusion pressure and the cortical electroencephalogram following concussive brain injury in cats (16). Although these authors did not measure plasma or regional brain opioid peptides, their data are the first to suggest that endogenous opioid substances may be released

after experimental closed head injury. Studies from our laboratory, supported by the present contract, have recently demonstrated that the κ-selective opiate antagonist WIN44,441-3 stereospecifically improves blood pressure and focal cerebral blood flow following closed head injury (17). No studies to date, however, have examined the role of specific endogenous opioid systems, particularly dynorphin, in mediating the cardiovascular changes following head trauma.

Thryotropin-releasing hormone (TRH) has been tested in spinal cord injury for the same reasons it was evaluated in experimental shock, based on its ability to act in vivo as a partial physiological antagonist of endorphin systems. Not only was TRH found to be beneficial in treating traumatic spinal cord injury in the cat, but TRH proved significantly superior to both naloxone and high-dose corticosteroids in this regard (18). The beneficial effect of TRH on motor recovery following traumatic cervical injury in the cat was dose related, with significant actions noted at doses as low as 0.02 mg/kg (as IV bolus plus four-hour IV infusion; total dose, 0.1 mg/kg). In addition, TRH proved to be effective, even when treatment was delayed as long as 24 hours after injury (18).

Given the obvious similarities between pathophysiological consequences of head injury and spinal does injury, it is somewhat surprising that so little experimental work in head injury has been performed to date. In a single report, TRH treatment has been found to improve neurological function and EEG following brain stem compression in the cat (19). This study and the results obtained from spinal cord injury studies suggest a potential therapeutic role for TRH in cerebral injury.

APPROACH TO PROBLEM

Our technical objectives under the current contract are (a) to evaluate changes in endogenous opioids after traumatic brain injury; (b) to characterize the physiological response to graded levels of traumatic brain injury, including mean arterial pressure, intracranial pressure, brain perfusion pressure, cerebral blood flow, electroencephalographic changes, and brain stem auditory evoked responses; (c) to examine the therapeutic effects of selective κ-opiate receptor antagonists and analogs after experimental head injury; (d) to determine whether more selective opiate antagonists or TRH analogs are more effective than naloxone in the treatment of closed head injury; (e) to compare the effects of the various pharmacological treatments on blood flow and physiological changes following traumatic brain injury.

A. CAT STUDIES

Brain injury was induced by a fluid percussion device that causes graded brain injury through brief distortion of neural tissue (20). Sixty male or female cats (3.0 - 3.5 kg) were anesthetized with sodium phenobarbital (50 mg/kg), paralyzed with a continuous infusion of pancuronium (0.6 mg/kg/h) and artificially ventilated throughout the experiment with 70% nitrous oxide and 30% oxygen using an Omni Veterinarian Anesthesia Machine (Ohmeda Corporation, San Rafael, CA) and a Harvard ventilator (Harvard Apparatus, Milton, MA). Drugs were administered through a cannula placed in the inferior vena cava via the femoral vein. The femoral artery was cannulated (PE90) to monitor heart rate, mean arterial pressure (MAP) and pulse pressure (PuP) as well as to sample arterial blood gases. Prior to injury, sodium bicarbonate (0.16 mEq/ml) was administered as required to maintain pH within the normal range. With the animal in a stereotaxic frame, the scalp and temporal muscle were reflected and a hollow 17.5 mm "trauma" tube rigidly fixed with dental acrylic to the animal's

skull over an 18.0 mm craniotomy centered over the sagittal sinus, midway between lambda and bregma.

Mechanical deformation of the brain was produced by a fluid-percussion device, initially developed as a clinically relevant animal model of brain concussion (20,21). This device is a refinement of earlier fluid-percussion head injury models developed in other laboratories (21) and produces graded levels of brain injury associated with sudden deformation of neural tissues. Brain deformation results from the introduction of small volumes of fluid into the epidural space of the closed cranium by a metal pendulum which strikes the piston of the device from a predetermined height, forcing a fixed volume of isotonic saline through the hollow "trauma" tube into the skull cavity. The device produces a pulse of increased intracranial pressure (ICP) of fairly constant duration (21-23 msec). Since increasing fluid loading produces greater magnitudes of injury, the magnitude of injury is regulated by varying the height of the pendulum.

A piezoelectric pressure transducer affixed to the injury device measured the pressure transient which was recorded on a storage oscilloscope (Tektronix) and photographed with a Polaroid camera. The duration and peak pressure in atmosphere (atm) was noted for each injury.

Intracranial Pressure and Mean Arterial Blood Pressure

A small hole (7.0 mm in diameter) was drilled in the skull, and the dura was exposed in order to place a plastic cannula to record intracranial pressure (ICP). The exposed dura was cut so that the cerebrospinal fluid had unobstructed access to the cannula. The hole was sealed with dental acrylic, and the cannula was filled with normal saline. This method of monitoring ICO has been used successfully in many previous studies of fluid-percussion injury (21,23) since insertion of an intracerebroventricular cannula can cause additional tissue injury when fluid-percussion is initiated. Changed in MAP and

ICP were monitored by strain gauge transducers, the outputs of which were recorded on a Narco Biosystem NT-40 polygraph (Narco Corporation, Houston, Texas). Arterial blood gases were analyzed on an IL Model 213 pH and blood gas analyzer (Instrumentation Laboratories, Lexington, MA).

Electroencephalography (EEG)

During surgery, aluminum screws (2.56 mm x 3/8) were placed over the left and right parietal cortices for EEG recording. Fast-Fourier transformed computerized electroencephalogram (FFT-EEG) were recorded continuously on a Neurotrac Computerized EEG (Interspec, Inc., Philadelphia, PA). Left and right "raw" EEG, compressed spectral array (CSA), spectral edge (the EEG frequency below which 95% of all EEG activity occurs) and EEG amplitude (as measure by power band analysis) were continuously recorded over the duration of the study. EEG amplitude was measured in picowatts with multiple filter range set at 160 microvolts over 4 second epochs. Compressed spectral array was measured over 4 second epochs with sensitivity set at 1 to 30 Hz (spectral edge was set at 95% of total power).

Cerebral Blood Flow

The radioactive microsphere technique was used to measure CBF because the technique allows for repeated measurements of regional CBF with the same animal (23). The radionuclides used for these studies were niobium-95, gadolinium-153, strontium-85, scandium-46, and tin-113; all had a specific activity of 10 mCi/g. ^{85}Sr, ^{46}Sc, ^{95}Nb, ^{153}Gd, were obtained from 3M, New Brighton, Minnesota, and 113Sn from New England Nuclear, Boston, Massachusetts. Experiments were carried out on 16 cats, anesthetized as previously described. A cannula (PE90) was also placed in the left femoral artery for withdrawal of reference arterial samples, A PE90 cannula with a slightly flared end was placed in the left atrium via a thoracotomy (see reference 6), and the chest was sutured closed.

For each CBF determination, 0.9 to 1.8 x 10^6 microspheres (15μm in diameter) in dextran and polyethylene sorbitan mono-oleate (Tween 80) were injected at 5 time points: prior to injury (baseline), 10 min post-injury (prior to drug administration), 30 min; 1 h and 2 h after injury. Following agitation for 4 min on a vortex mixer, microspheres were injected into the left atrium over approximately 30 sec. The injection of this number of microspheres insured that tissue samples over 250 mg would contain at least 400 microspheres (23). Just before and for 90 sec after each microsphere injection, reference arterial samples were withdrawn from the left femoral artery at a rate of 0.6 ml/min using Gastight syringes (Hamilton Company, Reno, Nevada) and a Harvard withdrawal pump (Harvard Apparatus, South Natick, Massachusetts). Counts from the arterial reference samples were used to calculate CBF.

After the final microsphere injection the animals were killed by transaortic perfusion with 0.9% NaCl solution followed by aldehyde fixatives. The brains were removed, sectioned coronally, and the frontal cortex, striatum, hippocampi, diencephalon, parietal cortex, midbrain/thalamus were dissected bilaterally, Pons, medulla and cerebellum were also dissected, This procedure yielded tissue samples varying in weight from 250 mg to 1.5 g. Tissue samples were weighed and counted, along with the arterial reference samples in Beckman Gamma 300 gamma counter (Beckman Instruments). Matrix inversion and CBF calculations were performed using a Datapoint 1800 minicomputer. Cerebral blood flow was calculated using the following equation (6,33):

$$\text{CBF (ml/100 g/min)} = \frac{Cb \times 100 \times RBF}{Cr}$$

where Cb indicates counts in brain, Cr indicates counts in arterial samples, and RBF us the arterial withdrawal rate.

Radioimmunoassays

In a separate group of animals brains were removed, cooled in ice cold sucrose and dissected according to the following scheme: frontal cortex, parietal cortex, striatum, hippocampus, midbrain, thalamus, pons, medulla and cerebellum.

For radioimmunoassay, tissue samples were stored frozen in closed tubes at -70°C until extraction by heating in 250 µl 0.1 M acetic acid at 95°C for 10 min. The samples were sonicated for 15 sec, and an aliquot was taken for protein determination by a modification of the Lowry method. Triton X-100 was added to the suspension, to a final concentration of 0.1% and the mixture was centrifuged at 10,000 g for 3 min. Duplicate aliquots of the supernatant were taken for taken for radioimmunoassay. Immunoreactive dynorphin A (Dyn-$\underline{ir}$) was determined using the "Lucia" antiserum (kindly provided by Dr. Avram Goldstein) which recognized dynorphin 1-17 and dynorphin 1-12, as well as larger putative precursors of dynorphin. Dynorphin 1-11, dynorphin 1-10 and dynorphin 1-9, α-neo-endorphin, enkephalin, β-endorphin and shorter fragments of dynorphin have no significant cross-reactivity. Immunoreactive leu-enkephalin (Enk-$\underline{ir}$) was determined using the "Llugh" antiserum (kindly provided by Dr. Gregory Mueller). An IC_{50} of approximately 20 fmoles/assay (65pmol) was determined for leu-enkephalin. The cross-reactivity of this antiserum was less than 0.5% for met-enkephalin, dynorphin 1-8, dynorphin 1-17 and dynorphin 1-13, less than 0.1% for α-neo-endorphin and β-endorphin, and less than 0.01% for met-enkephalin-Arg-Phe and β-endorphin. Radioactivity of the pellets was determined in a Micromedic Systems 4/6000 gamma counter with data reduction performed by a Hewlett-Packard 9815-A electronic calculator using log-logit transformation.

Gross Pathology

Upon brain dissection for peptide analysis or regional CBF studies, the occurrence of regional tissue hemorrhage and/or necrosis were recorded for animals treated with WIN(-) (n=10), WIN(+) (n=10) or saline (n=10). Scoring for post-injury pathological tissue damage was performed in a blinded fashion, and was based on the following scale; 0 = no observable damage; 1 = slight hemorrhage present; 2 = moderate hemorrhage present; 3 = severe hemorrhage and/or necrosis present.

EXPERIMENTAL PROTOCOL

Experiment 1

In order to examine the role of specific endogenous opioid receptors in traumatic brain injury, 15 min following brain injury of similar magnitude (3.2 - 3.6 atm), one group of animals received either: (1) the opiate antagonist WIN44,441-3 (WIN(-)) which has increased activity at κ-sites (43) (0.2 mg/kg in 10 cc saline, n=12); (2) its dextrostereoisomer WIN44,441-2 (WIN(+)), which is inactive at opiate receptors (0.2 mg/kg in 10 cc saline, n=12); or (3) saline vehicle (10 cc, n=12) in a double-blinded fashion. The dose chosen was based upon previous work in our laboratory relative to spinal trauma and ischemia (15). To evaluate whether potential effects of the WIN(-) compound were dependent solely upon its action on blood pressure, a fourth group of animals were treated with a constant infusion of dopamine hydrochloride alone (20 µg/kg/min, n=6) beginning 15 min following injury. Regional cerebral blood flow (CBF) was measured in a subgroup of animals using the radiolabelled microsphere technique as described above. Sequential measurements of CBF were performed 10 min prior to injury (baseline) and 10, 30, 60 and 120 min after injury in animals receiving WIN(-) (n=5), WIN(+) (n=5), saline (n=3), or dopamine (n=3).

Experiment 2

In order to correlate changes in systemic and cerebral cardiovascular indices with regional changes in brain opioid peptide concentrations, one group of animals (n=12) was anesthetized, surgically prepared and sacrificed prior to injury in order to obtain pre-injury (baseline) values. A separate group of animals was surgically prepared and either injured (3.2 - 3.6 atm, n=12) or maintained on inhalation anesthesia (anesthesia controls) for 2 h (n=6). All animals were sacrificed a 2 h, brains removed, dissected and analyzed for regional opioid peptide concentrations (dynorphin 1-17 and leu-enkephalin) by radioimmunoassay as described above.

Data Analysis

All data are expressed as mean $\pm$ S.E.M. Statistical analyses were performed employing parametric analyses of variance (ANOVA) for repeated measures followed by Duncan's multiple range test. For cerebral blood flow, statistical comparisons were carried out using analysis of covariance and Duncan's multiple range test. Kruskal-Wallis ANOVA was used to compare all ordinal data. Fisher's Exact Probability Test was used to compare survival data. A `p' value < 0.05 was considered statistically significant.

RESULTS

MAP and ICP

Mean arterial pressure in all animals increased approximately 90 mm Hg (from 143 ± 7 to 230 ± 9 mm Hg) by 2 min post-injury and remained elevated for up to 5 min. By 15 min post-injury (immediately prior to drug administration), MAP had returned to control valued. Subsequently, MAP in all saline- and WIN(+)-treated animals continued to fall, reaching hypotensive levels (as compared to baseline: MAP $\leq$ 90 mm Hg) by 2 h post-injury. Administration of WIN(-) at 15 min following injury reversed the fall in MAP within 5 min. WIN(-)-treated animals had significantly higher MAP than WIN(+)- or saline-treated animals within 5 min of drug administration (p $<$ 0.05). MAP in

Page 14

the WIN(-) group remained significantly elevated when compared to WIN(+)- or saline-treated animals (p < 0.01) over the remainder of the 2 h monitoring period. A transient increase in ICP occurred but ICP returned to normal by 10 min after injury. Administration of WIN(-), WIN(+) or saline had no effect on post-traumatic intracranial pressure.

EEG

Fluid-percussion injury caused a rapid fall in EEG amplitude, as measured by power band analysis, to 36 ± 5% of control levels (p > 0.001) by 1 min post-injury and to 32% (p < 0.001) of control by the end of the 2 h study period in WIN(-)- or saline-treated animals. In over 40% of all animals following injury, the diminution of EEG amplitude was so marked following injury that the CSA spectral edge was entirely abolished.

Administration of WIN(-) caused a significant increase in EEG amplitude within 5 min of drug treatment (p < 0.001). By 15 min after drug treatment, EEG amplitude had returned to 80% of control values (p < 0.001) this increase was maintained throughout the remainder of the 2 h observation period. Following administration of WIN(-), the significant increase in EEG amplitude was also accompanied by a restoration of the EEG waveform and spectral edge. Administration of WIN(+) was without effect on restoring the spectral edge in those animals.

Regional CBF

Traumatic brain injury in saline-treated animals caused either a slight increase or no change in regional CBF by 10 min post-injury. Regional CBF began to decline by 30 min post-injury and 2 h post-injury was significantly depressed in the frontal cortex (-29%; p < 0.05), striatum (-43%; p < 0.05), hippocampus (-50%; p < 0.05), midbrain/thalamus (-40%; p < 0.05), pons (-39%; p , 0.05) and medulla (-48%; p < 0.05). A significant decrease in whole brain blood flow was

also observed in saline-treated animals at 2 h post-injury (from 46 ± 8 baseline to 31 ± 5 ml/100 g/min, p , 0.05).

In animals treated with WIN(+) 15 min after injury, whole brain blood flow continued to decline after injury and was significantly depressed at 2 h post-injury (x = 37 ± 5 ml/100 g/min, p < 0.05; Table 3). Regional CBF declined maximally in WIN(+)-treated animals at 2 h post-injury in those areas demonstration greatest pathological changes on gross examination: striatum (-29%; p < 0.05); midbrain (-41%; p < 0.05); frontal cortex (-28%; p < 0.05); pons (-33%; p = nonsignificant); medulla (-29%; p < 0.05); and cerebellum (-42%; p< 0.05).

Administration of WIN(-) at 15 min post-injury caused a significant increase in whole brain blood flow at 2 h post-injury (from 44 ± 4 to 56 ± 7 ml/100 g/min; p < 0.05). Regional blood flow measured at 2 h post-injury was also significantly increased by WIN(-) treatment when compared to post-injury values; frontal cortex (+47%; p < 0.01), striatum (+36%; p < 0,05), midbrain (+37%; P < 0.05), pons (+25%; p = nonsignificant).

Dopamine Treatment

Constant infusion of dopamine alone (n=5) caused a significant and prolonged elevation in MAP (mean increase = 32 mm Hg), which paralleled that of WIN(-). However, no improvement of the FFT-EEG was noted following dopamine treatment. Animals treated with dopamine (n=3) showed no improvement in regional CBF following injury. Regional CBF continued to decline after injury in dopamine-treated animals and by 2 h was significantly depressed in frontal cortex (-35%; $p < 0.05$), striatum (-38%; $p < 0.05$), and brainstem (-37%; $p < 0.05$).

Survival

Among control animals, 6 of 12 animals treated with WIN(+), 5 of 12 animals treated with saline and 2 of 6 animals treated with dopamine died within the 2 h study period after injury. All saline- and WIN(+)-treated animals died when they became severely hypotensive. In contrast, all 12 animals receiving WIN(-) survived following trauma. This difference in survival between WIN(-) and control (saline- or WIN(+)-treated) animals was statistically ($p = 0.04$, Fisher's Exact Probability Test).

Gross Pathology

Upon brain dissection, it was observed that fluid-percussion injury in saline-treated animals caused reproducible intraparenchymal hemorrhage in the midventral aspect of the pontomedullary junction (100% of animals), frontal cortex (80% of animals), striatum (80% of animals), midbrain/thalamus (100% of animals), hippocampus (50% of animals), hypothalamus (80% of animals), pons (100% of animals) and medulla (100% of animals). This pattern and severity of intraparenchymal hemorrhage was similar to that observed in animals treated with WIN(+). However, in the WIN(-)-treated animals, the severity and incidence of hemorrhage was reduced in frontal cortex (65% of animals), midbrain/thalamus

(50% of animals). The severity of hemorrhage was also found to be reduced in pons and medulla of WIN(-)-treated animals.

Experiment 2

At 2 h following injury, significant changes were observed in regional concentrations of Dyn-ir, when compared to uninjured valued. A significant decline (78%; $p < 0.01$) was found in Dyn-ir of the anterior pituitary at 2 h post-injury. Conversely, significant regional increases of Dyn-ir were noted in frontal cortex (66%; $p < 0.01$), striatum (43%; $p < 0.05$), hippocampus (40%; $p < 0.05$), midbrain/thalamus (35%; $p < 0.05$), pons (57%; $p < 0.05$) and medulla (53%; $p < 0.05$). Regional concentrations of Enk-ir were not significantly changed at 2 h following injury. The increases in regional concentration of Dyn-ir occurred predominantly in those areas showing greatest decreases in regional cerebral blood flow following injury in control animals. Regional concentrations of Dyn-ir and Enk-ir in anesthesia/surgical control animals did not differ from those obtained from uninjured animals.

B. RAT STUDIES

During the second year of the present contract, we have successfully established two new traumatic head injury models in the rat, based upon the same fluid percussion technology we have used in the cat; a midline (vertex) and lateral (parasaggital) model. A reproducible injury curve has been generated in our laboratory, based on chronic (4-week) post-injury neurological scores. These neurological scoring tests, which have been developed in our laboratory, appear to be sensitive to small changes in the level of severity of trauma. We have utilized the lateral (parasaggital) model (see Annual Report Year 1 of Contract for methodological details) to evaluate the therapeutic effects of 1) nalmefene, a newly synthesized opiate antagonist with increased affinity at κ-receptors and 2) CG-3703, a long-acting centrally active analog of

thyrotropin-releasing hormone (TRH). Nalmefene was given priority over WIN(-) in these studies since it is a newer generation of opiate antagonists with enhanced affinity for κ-opiate receptors.

Methods

Forty male rats weighing from 400 - 500 g were initially anesthetized with ketamine (80 mg/kg, i.m.) and sodium pentobarbital (20 mg/kg, i.p.). during surgical preparation and throughout the experimental, all wounds were infused with a topical anesthetic (lidocaine hydrochloride, 2.0%). A bilateral femoral cutdown was performed and femoral venous (for drug administration) and arterial (for blood pressure/blood gas monitoring) catheters were inserted. With the animal in a stereotaxic frame, the scalp and temporal muscle were reflected. Next, a hollow female Leur-Loc fitting 2.0 mm (to be used to induce trauma) was rigidly fixed with dental cement to the animal's skull in a craniotomy centered over the left parietal cortex 5 mm form lambda, 5 mm from bregma, 4 mm from sagittal suture. The dura was left intact at this opening. Stainless steel screw electrodes were inserted into the skull over the right and left parietal cortices (recording electrode) and the anterior nasal bone (reference electrode) to record electroencephalographic (EEG) tracings.

Fluid-percussion Injury

The fluid-percussion device used to produce experimental brain injury was identical to that which has been used in cats and described in the previous Annual Report (Year 1). Briefly, it consisted of a Plexiglas cylindrical reservoir, 60 cm long and 4.5 cm in diameter, bounded at one end by a Plexiglas, cork-covered piston mounted on O-rings. The opposite end of the reservoir was fitted with a 2 cm long metal housing on which a transducer (Gould, Inc.) was mounted and connected to a 5mm tube (2mm inner diameter) that terminated with a male Leur-Loc fitting. At the time of injury the tube was connected to a female Leur-Loc fitting that had been chronically implanted over the exposed cortex of

the rat. After the entire system was filled with 37°C isotonic saline, injury was induced by a metal pendulum which struck the piston of the device from a predetermined height. The device produces a pulse of increased intracranial pressure (ICP) of fairly constant duration (21 - 23 msec) by injecting varying volumes of saline into the closed cranial cavity. Brief displacement and deformation of neural tissue results from the rapid epidural injection of saline and increased magnitude of tissue deformation is associated with an increased magnitude of brain injury. The magnitude of injury was regulated by varying the height of the pendulum, which resulted in corresponding variations in intracranial pulses expressed in atmospheres (atm). These pressure pulses were measured extracranially by transducer (housed in the injury device) at the time of injury, recorded on a storage oscilloscope and photographed with a Polaroid camera.

Physiological Monitoring

Systolic, diastolic and mean arterial pressure (MAP) were recorded continuously before and after head injury via the femoral artery catheter. Pressures were monitored by strain gauge transducers, the output of which were recorded on Narco-Biotrace-40 physiograph. Arterial blood gases (pH, PO_2, pCO_2) were analyzed at regular intervals throughout the experiment using a Corning pH and blood analyzer.

For laboratory studies, the EEG electrodes were connected to a Neurotrac Systems Computerized Spectral EEG Analyzer in order to obtain continuous pre- and post-injury Fast Fourier-Transformed (FFT) spectral EEG recordings including Fourier-Transformed compressed spectral array (CSA), spectral histogram and frequency/amplitude (power band) analysis.

Experimental Protocol

Immediately following surgical preparation, a constant i.v. infusion of sodium pentobarbital (15 mg/kg/h) was begun. During a 2-h baseline period, MAP

and EEG were continuously recorded and arterial blood gases monitored. At the end of the 2-h baseline period, animals were attached to the fluid-percussion device and injured at an injury level of 1.9 - 2.2 atmospheres (atm). At $t = 30$ min, each animals received an i.v. bolus of either: (1) TRH analog CG-3703 (1.0 mg/kg, $n = 10$); (2) nalmefene (0.1 mg/kg, $n = 10$); or (3) saline ($n = 20$). MAP and EEG were monitored continuously for 2 h post-injury. At $t = 2$ h, i.v. pentobarbital infusion was turned off and animals were returned to their home cages. Ten additional animals were identically prepared but were not injured and served as sham controls.

Neurological Evaluation

Chronic neurological scoring of motor function was performed daily in all animals for a 4-week period. Neurological function was evaluated by a trained observer who was unaware of each animal's level of injury using ordinal scale. Animals were scored on a 4 (normal) to 0 (severely impaired) scale using each of the following indices: (a) forelimb flexion upon suspension by the tail; (b) decreased resistance to lateral pulsion; (c) circling behavior upon spontaneous ambulation; (d) ability to stand on an inclined angle board with the maximal angle at which the animal can stand for 5 sec recorded (angle board) where 45 - $50° = 4$, 40 - 45 ° = 3, 35 - 40 = 2, 30 - 35° = 1, 0 - 29° = 0. A total composite functional neurological score (0 -20) was obtained by combining the scores for the several tests of motor function so that 20 - normal, 15 - slightly impaired, 10 = moderately impaired, 5 = severely impaired, 0 = afunctional.

Continuous variables compared across groups were examined utilizing analysis of variance (ANOVA) followed by Newman-Keuls tests. Continuous variables subjected to repeated measurements over tine (e.g., cardiovascular) were subjected to repeated measurement ANOVA followed by Dunnett's test at each time point. Ordinal measurements such as neurological scores were evaluated

utilizing the non-parametric Kruskall-Wallis ANOVA followed by Dunnett's tests at each time point. Ordinal measurements such as neurological scores were evaluated utilizing the non-parametric Kruskall-Wallis ANOVA followed by individual non-parametric Mann-Whitney U-tests. Correlations between continuous physiological and neurochemical variables were made utilizing Spearman's Rank Correlation method. Survival differences were compared using Fisher's Exact Probability Test. A 'p' value < 0.05 was considered statistically significant.

Cardiovascular Variables

Fluid-percussion brain injury of moderate severity produced a significant hypertensive response (from 93 ± 2 to 152 ± 4; $p < 0.05$) which returned to baseline by 5 min. By 30 min post-injury (immediately prior to drug administration), MAP had returned to baseline levels (94 ± 4 mm Hg). MAP increased significantly immediately following administration of CG-3703 to reach maximal levels by 15 min post-treatment ($x = 117 \pm 3$ mm Hg; $p < 0.05$ when compared with baseline). MAP continued to remain significantly elevated in the TRH analog-treated group for the duration of the 2-h study period ($x = 112 \pm 2$ mm Hg at 2 h). MAP of nalmefene-treated animals was significantly elevated at 45 min when compared to saline-treated animals ($x = 107 \pm 4$) and remained elevated for the duration of the study. MAP of saline-treated animals remained slightly below baseline values for the duration of the study ($x = 87 \pm 2$ mm Hg).

Electroencephalogram

Following brain injury in saline-treated animals, the compressed spectral edge of the EEG from the injured hemisphere decreased within the first minute (from 16 ± 0.2 to 9 ± 0.2 Hz; $p < 0.05$) and remained significantly depressed over the 2-h study period ($x = 12 \pm 0.1$ mm Hg at 2 h; $p < 0.05$, Fig. 2). No changes were observed in the compressed spectral edge recorded from the contralateral (uninjured hemisphere). Administration of TRH or nalmefene had no effect on compressed spectral edge post-injury. EEG amplitude (as measures by

total power band analysis in the injured hemisphere fell precipitously by 1 min post-injury to reach a nadir by 5 min (4% baseline; $p < 0.001$). At $t = 30$ min, EEG amplitude increased and by 1.5 h post-treatment had returned to 50% baseline. EEG amplitude in saline-treated animals remained at 20% baseline for the duration of the study. When each individual frequency was analyzed separately (power spectral analysis), it was discovered that administration of CG-3703 caused a two-fold increase in alpha-activity (from 10% baseline at 30 min post-injury to 33% baseline at 2 h post-injury; $p < 0.05$). Similar effects on individual EEG frequencies were not observed following nalmefene or saline administration.

Chronic Neurological Outcome

At 24 h and 1 week post-injury, saline-treated animals exhibited a severe neurological impairment. By 2 weeks post-injury, all saline-treated animals exhibited a moderate neurological deficit which persisted up to 4 weeks following injury. In contrast, by 24 h, CG-3703-treated and nalmefene-treated animals showed a significantly better neurological outcome when compared to saline-treated animals ($p < 0.05$). By week 1 post-injury, animals treated with CG-3703 or nalmefene exhibited only a mild neurological deficit. By the second post-injury week, however, all CG-3703- and nalmefene-treated animals were normal with respect to baseline neurological scores and significantly improved when compared to saline-treated animals tested at comparable times post-injury ($p < 0.01$).

Survival

Fifty-eight percent of all saline-treated animals ($n = 7$) died within 2 days following fluid-percussion brain injury. In contrast, all animals treated with either CG-3703 or nalmefene survived up to 4 weeks post-injury. This difference in survival was significant (Fisher's Exact Probability Test; $p = 0.05$).

Magnetic Resonance Spectroscopy: Pilot Studies

Male rats (n = 29) weighing from 350 - 450 g were initially anesthetized with ketamine (80 mg/kg, i.m.) and sodium pentobarbital (20 mg/kg, i.p.). Femoral venous and arterial catheters were implanted using polyethylene 50 tubing. With the animal in a stereotaxic frame, the scalp and temporal muscle were reflected. A small burr hole was placed over the left parietal cortex and a 2 mm hollow female Leur-Loc was rigidly fixed with dental cement to the animal's skull. Following surgery, anesthesia was maintained by continuous infusion of sodium pentobarbital (15 mg/kg/h). Experimental brain injury was induced using a lateralized, fluid-percussion model as previously described.

^{31}P MRS spectra were obtained using a 2 tesla GE CSI spectrometer operating at the phosphorus resonant frequency (34.6 MHz). Each animal (n = 10) was placed in a specially designed Plexiglas holder and positioned in the center of the magnet bore. A two-turn 5 x 9 mm NMR coil was placed centrally around the trauma site and used to transmit and receive signal. Skin and temporal muscle was retracted well clear of the trauma site to ensure that there was no contribution from there tissues to the ^{31}P MRS brain spectrum. Magnetic field homogeneity was optimized by shimming on the proton resonance of tissue water. ^{31}P MRS spectra were obtained in 10 min blocks prior to and for 8 h following injury, using a 90° pulse and 7-- msec repetition rate. Injury (1.5 - 2.5 atm) was induced either (a) outside the magnet, and the animals replaced in the holder and repositioned in the magnet bore; or (b) directly inside the magnet bore using a 1.5 m non-compliant lexan extension tube attached to the percussion device. The purpose of method (b) was to minimize the time difference between impact and ^{31}P MRS data accumulation while at the same time using lexan to ensure that there was no attenuation of the saline pulse passing through the extension tube. Neurological, physiological and MRS results using both procedures were identical.

The accumulated free induction decays (spectral width 4000 Hz, 2048 data points) were analyzed following zero filling, application of a 25 Hz Gaussian filter, and Fourier Transformation. The broad component of the spectra, assigned to relatively immobile phosphate residues in bone and phospholipid was removed mathematically by convolution difference. The relative changes in individual metabolite concentrations were determined using the GE computer program (GEMCAP), which integrated the area of individual peaks in the spectra following a "line fitting" procedure. By comparing each measured signal intensity with the animals pre-injury spectrum, each animal served as its own control.

The rapid repetition rate used in these experiments was empirically determined so as to give the best signal to noise ratio in the time resolution required. However, when determining metabolite ratios, saturation effects through inadequate relaxation times must be taken into account. Since phosphocreatine (PCr) and inorganic phosphate (Pi) have low and similar relaxation times, their saturation effects are similar and the error in their ratio is only 10%. Accordingly, we have used the PCr/Pi ratio as the chosen indicator of cellular bioenergetic status. Intracellular pH was calculated using the equation:

$$pH = 6.77 + \log(\Delta Pi - 3.29)/(5.68 - \Delta Pi) \ldots \ldots (1)$$

where Δ Pi is the chemical shift of Pi relative to PCr.

Systolic, diastolic and mean arterial blood pressure (MABP) were recorded continuously before and after head injury via the femoral artery catheter. Pressures were monitored by strain gauge transducers, the output of which were recorded on a Narco Biotrace-40 physiograph. Arterial blood pH and gases (pO_2,

pCO_2) were analyzed at 30 min intervals throughout the experiment using a Corning pH and blood gas analyzer.

RESULTS

Spectral assignments were made according to previous work on brain. The Pi peak was initially located at 4.91 ± 0.05 ppm (n = 10, mean ± S.D.), which corresponds to an intracellular pH of 7.10 ± 0.03. This value of brain intracellular pH is in good agreement with previously published values. Immediately following injury, the Pi peak shifted downfield (to the right) indicative of a more acidic environment. This acidic shift of the Pi peak was transient in nature, the lowest pH recorded being 6.86 ± 0.11 (p < 0.05) at 40 min following trauma, followed by a return of brain intracellular pH to pre-injury levels by 90 min.

The PCr/Pi ratio also demonstrated a transient change with time that correlated with the transient changed in intracellular pH. By 40 min post-injury, the PCr/Pi ratio had fallen from a pre-injury value of 4.8 ± 0.4 to 2.8 ± 0.7 (p < 0.05), then returned to approximate control levels by 90 min post-injury. Thereafter, the PCr/Pi ratio decreased again, independent of any pH changes, to a value of 2.3 ± 0.5 by 4 h post-injury (p < 0.01 when compared to pre-injury baseline levels), and remained suppressed for the duration of the 8 h experiment. There were no significant changes in ATP levels accompanying either the changes in pH, or the fluctuation in PCr/Pi ratio.

Despite the presence of hemorrhage within the MRS sensitive volume we do not believe that the presence of blood made a significant contribution to the ^{31}P MRS spectra based on the following: no significant 2,3-diphosphoglycerate peak, which is a major identifying resonance in blood, was apparent in our spectra. Furthermore, the presence of pooled blood would result in a significant Pi peak being present at an acidic chemical shift. This was not evident in our post-injury spectra, in which the 2 to 8 h spectra reflected a post-trauma pH of greater than 7.1.

CONCLUSIONS

The present set of studies demonstrated that dynorphin- but not leu-enkephalin-like immunoreactivity accumulates in injury regions after traumatic head injury; that such changes correlate with localized decreases in CBF in saline-treated animals; and that the somewhat κ-selective opiate antagonist WIN44,441-3 significantly improved mean arterial pressure, electroencephalographic activity, and localized cerebral blood flow following traumatic head injury in the cat. The beneficial effects of WIN44,441-3 were clearly stereospecific, since administration of its stereoisomer WIN44,441-2 had no effect on any physiological parameters following traumatic head injury. The observation that the beneficial effects of the WIN compound were stereospecific strongly support our hypothesis that the therapeutic efficacy of opiate antagonists in experimental head injury are through actions at opiate receptors. The beneficial effects of WIN(-) were found at doses significantly lower than those shown to be effective with naloxone, further suggesting that the effects may be mediated through a κ-receptor mechanism of action. Our studies are the first to show an improvement in brain cerebral blood flow following administration of opiate antagonists in traumatic brain injury. This improvement of blood flow to specific brain regions such as midbrain, brainstem and basal gangiia following administration of the opiate antagonist, suggests that opioid peptides released following head injury may act at κ-receptors to diminish brain blood flow. Our results employing opiate receptor antagonists in the cat were strengthened by the results we obtained utilizing opiate receptor antagonists and TRH analogs in the lateralized head injury model in the rat, since we observed that centrally active TRH analog CG-3703 and the newly synthesized opiate antagonist nalmefene improved MAP, neurological outcome and survival following traumatic brain injury in the rat.

Finally, we have demonstrated the potential usefulness of nuclear magnetic resonance spectroscopy (MRS) in the non-invasive evaluation of biochemical changes following brain injury. Using phosphorus MRS, we have examined certain biochemical responses of rats over an 8 h period following lateralized fluid-percussion brain injury (1.5 - 2.5 atmospheres). Following injury, the ratio of phosphocreatine to inorganic phosphate (PCr/Pi) showed a biphasic decline: the first decline reached its nadir by 40 min post-trauma with recovery by 100 min, followed by a second decline by 2 h that persisted for the remaining 6 h observation period. The first, but not the second, decrease in PCr/Pi was associated with tissue acidosis. No changes in ATP occurred at any time during the injury observation period. It is clear that such changes may be indicative of altered mitochondrial energy production following brain injury, which may account for the reduced capacity of the cell to recover from traumatic injury.

RECOMMENDATIONS

Our recent studies, combined with previous studies from our laboratory, provide the experimental basis for the evaluation of opiate antagonists in clinical head injury. Selective opiate antagonists such as WIN44,441-3 and nalmefene, or TRH analogs may ultimately prove to have advantages over naloxone in the treatment of traumatic brain injury. New and more highly selective κ-receptor antagonists have been developed (bi-naltorphamine) and novel long-acting TRH analogs have been recently made available for experimental use. Such drugs should be studied in experimental models of head injury and experiments should be directed toward understanding their mechanism of action. Finally, magnetic resonance spectroscopy/imaging should be further evaluated for its non-invasive prognostic abilities in head injury by identifying the extent and degree of injury (MRI), as well as to monitor metabolic changes within the injured tissue (MRS). Such techniques may also prove to be particularly useful for evaluating the possible therapeutic effects of interventional therapies.

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